Behavioural Responses of Broiler Chickens during Low Atmospheric Pressure Stunning

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Abstract

Low atmospheric pressure stunning (LAPS) is a new irreversible stunning method for broiler chickens (*Gallus gallus domesticus)*, which has the potential to improve welfare during routine slaughter. During LAPS, birds are placed in a hypobaric chamber that allows oxygen to be gradually removed from the environment by the controlled removal of air; the staged process takes 280s and reaches final decompression pressure that is 80.6 kPa below atmospheric pressure (nominally 101.3 kPa for an absolute vacuum pressure of 20.7 kPa). In this study, the behaviour of broilers (50 individuals and 50 focal birds killed in groups of 20) was observed during LAPS. Latencies, total durations, single bout durations and number of bouts were recorded for all behaviours. Three different decompression curves were applied during the process (based on automatically applied settings related to ambient temperature) and their effects on behaviour were investigated. Not all birds displayed all behaviours, but a subset of behaviours (ataxia, loss of posture, clonic and tonic convulsions and leg paddling) occurred in a consistent sequence. In individuals, mandibulation, headshaking and open bill breathing occurred earliest at 44.5s ± 31.6s, 50.8s ± 38.3s and 57.4s ± 35.8s respectively after LAPS began. Ataxia was observed on average at 57.3s ± 11.5s, with birds killed at colder temperatures taking slightly longer to succumb to ataxia than those at warmer temperatures. Loss of posture (LOP) is regarded as a behavioural marker for loss of consciousness and it occurred on average at 80.7s ± 17.7s. Clonic and tonic convulsions were displayed after LOP at 110.5s ± 37.6s and 117.4s ± 28.8s after LAPS onset respectively. Mean time to motionless was 199.4s ± 21.3s. The group data were largely similar to that of individuals but were less reliable due to focal birds being obscured by neighbours. Based on LOP, the data suggest that birds are in a conscious state for longer during LAPS than in controlled atmosphere stunning with inert gases, but although the induction to unconsciousness is more gradual, other behavioural responses were equivalent. The occurrence of mandibulation, head shaking, and open bill breathing may be an indication of reduced welfare or may be indications of a non-painful physiological responses to hypoxia in a hypobaric atmosphere. These behaviours occurred at similar levels as seen in CAS with inert gases in poultry and the lack of escape

behaviours as well as absence of signs of severe dyspnoea suggest that LAPS is a humane approach to stunning of poultry.

Key words: Animal welfare, hypobaric hypoxia, humane slaughter, and loss of posture, low atmosphere pressure stunning

1. Introduction

Approximately 17.8 million broiler chickens (*Gallus gallus domesticus*) are killed in the UK every week (DEFRA, 2015), so welfare at the point of slaughter is an important issue. Electrical stunning is associated with various welfare concerns including shackling of conscious birds, pre-stun shocks and the risk of inadequate stunning (Raj, 2006). Recent EU legislation, Regulation (EC) no. 1099/2009 on the protection of animals at the time of killing (European Commission, 2009), provides stricter rules surrounding the use of electrical stunning which has fuelled increased uptake of controlled atmosphere stunning (CAS). While CAS has many welfare advantages (birds are not shackled while conscious; all birds are stunned), birds are not rendered unconscious immediately, which could potentially result in pain and suffering – if, for example, nociceptive concentrations of carbon dioxide were used (Raj, 2006; Shields and Raj, 2010). There has been much research on the welfare implications of CAS (reviewed in Raj, 2006) and most studies have focussed on identifying gas mixtures that result in the most humane stun. A related but novel approach, Low Atmospheric Pressure Stunning (LAPS), has been developed in the United States, in which birds are stunned by gradual decompression resulting in hypobaric hypoxia. Thus, during LAPS, air (and therefore oxygen) is gradually removed from the atmosphere, rendering the birds unconscious. LAPS is in routine commercial use at a poultry processing plant in Arkansas, having been given 'no objection' status by both the United States Department for Agriculture (USDA) in 2010 and the Canadian Food Inspection Agency in 2013.

Although rapid decompression is a source of welfare concern, it has been argued that gradual decompression can be humane (Vizzier-Thaxton et al., 2010). Previous research on LAPS identified

process variables for a suitably gradual decompression (Purswell et al., 2007), examined some aspects of behaviour and corticosterone responses (Vizzier -Thaxton et al., 2010), meat quality (Battula et al., 2008; Vizzier-Thaxton et al., 2010) and pathology (Vizzier-Thaxton et al., 2010). Other work examining hypoxia in poultry leading to anoxia achieved with a gas environment has reported favourable results for welfare (Woolley and Gentle, 1988; Raj et al., 1991), and supports the notion that LAPS could be a welfare friendly approach. The evidence from pilots exposed to hypobaric environments suggests that effects of slow decompression are specific to each individual and include gradual loss of cognition and motor skills without conscious awareness of the loss of these functions (Woodruff and Webb, 2011), though we note that care must be taken when making comparisons between humans and birds given important differences in their anatomy and physiology. Available evidence suggests that gradual hypoxia is promising as a humane method of stunning for poultry, but more research is required.

McKeegan et al. (2013) examined electroencephalogram (EEG) and electrocardiogram (ECG) responses of broilers undergoing LAPS using similar equipment and processes as used by Battula et al. (2008) and Vizzier-Thaxton et al. (2010). Application of LAPS was associated with changes in the EEG pattern in the form of highly significant increases in total power, decreases in mean frequency and in particular, progressive increases in slow wave (delta) activity, indicating a gradual loss of consciousness. ECG traces indicated an absence of heart rate elevation in the conscious period, suggesting that birds do not find LAPS induction distressing. However, the study was limited to one temperature range and 28 birds (due to the necessity of surgical implantation of EEG electrodes) and behaviour was not observed, so the suggested time to loss of consciousness of 40s has not been corroborated. Vizzier-Thaxton et al. (2010) incorporated some simple behavioural observations in their study of LAPS, in which behaviours indicative of anoxia were seen, but only ten replications of group observations were carried out. Detailed recordings of individual responses during LAPS is required to provide important information on whether and to what extent gradual decompression is associated with potentially negative behavioural responses. The primary objective of the study was to carry out a detailed behavioural analysis of broiler chickens undergoing LAPS, both in groups and individually, with a focus on behaviour occurring during induction to unconsciousness. The secondary

objectives were to investigate the effects of bird weight, and whether slightly adjusted decompression settings (automatically applied in relation to ambient temperature) had any effect on behavioural responses. Our aim was to create a timeline of behavioural events during LAPS and interpret this with regards to its welfare implications and EU legal requirements for animals to be spared any avoidable pain, distress or suffering during their killing and related operations.

2. Methods

2.1. Subjects and Husbandry

Fifty individuals and 50 groups of twenty commercial (mixed sex, as hatched) Ross 708 broiler chickens (*Gallus gallus domesticus*) were observed undergoing LAPS in two experiments. In the groups, one focal bird was observed in each LAPS run producing true replication. The birds were randomly selected from a single flock by a catching crew at normal depopulation and then randomly assigned to 50 groups of 21 (one of which was randomly selected to undergo LAPS individually). Individual birds were killed at 49 days of age and group birds were killed the next day. Individual birds were weighed and group weights were used to calculate means for birds subject to LAPS in groups. Bird weights were as expected in the US commercial system; at the time of killing they weighed 3.4 ± 0.5 kg (range 2.6-4.3 kg). The effects of gender could not be examined because the birds were from a commercial flock and were not sexed. Before both experiments, the birds were housed in 50 pens (1.22 x 1.22 m), either individually (with visual and auditory access to neighbours) or in groups for 24 hours before the trials. The pens had wood shavings litter and access to water and standard commercial diet. Before undergoing LAPS, the birds were feed restricted for eight hours and water restricted for two hours to mimic commercial practice, in which birds would normally be caught, transported and spend time in lairage without food and water. The last hour of each restriction took place in a standard US transport container (2.44 x 1.22 m) (immediately before LAPS). The trials were undertaken in Mississippi, USA, and therefore were not subject to UK legal requirements

through DEFRA or Home Office regulations. The experiments received ethical approval from the Animal Welfare and Ethics Committee of the School of Veterinary Medicine, University of Glasgow.

2.2. LAPS Process

The LAPS chamber was developed by Technocatch in Mississippi, USA and is used commercially to kill broilers for meat production. Technocatch has patented the system and the pressure curves applied by the process. The chamber used in the current study is a research unit, but is identical to those used commercially. The chamber is cylindrical (6.1-6.25 m in length and 2.13 m in diameter) and is designed to accommodate two standard US transport containers. The required decompression curve is automatically applied and controlled by a computer and once started, can only be stopped in the case of an emergency. A variable airflow withdrawal process controlled by pumps alters the atmosphere (Holloway, in prep). An infra-red camera (130^{o} camera with 18 infra-red illuminators, Model #RVS-507, RVS Systems) is fitted into every unit to observe the birds. A hydraulically operated door is present that allows the entry of the transport containers and seals them into the chamber to begin the process. The LAPS evacuation process takes exactly 280 seconds, after which the chamber is returned to atmospheric pressure using a baffled air inlet, prior to the door opening and the exit of the transport containers.

2.3. Temperature Settings

The temperature settings (pressure curves) are created automatically by a computer programme to control the extraction of O_2 from the environment. Because cold air is denser and therefore contains more oxygen than warm air and birds apparently respond differently to anoxia at different temperatures, slightly different pressure reduction curves must be applied to achieve the same hypobaric effect under different ambient conditions. As discussed by Holloway (in prep), water in the LAPS chamber may also lead to modification of the rate of decompression based on temperature. There are six temperature settings that are applied in accordance with ambient temperature and temperature settings 4, 3 and 2 were applied in this study; all the curves converge on a final pressure

of 20.7 kPa. The pressure curves of all temperature settings are identical until 67 s into LAPS; this is to avoid variability in decompression rate in the early stage of the process, which may have welfare consequences. The aim of the temperature settings is to have all birds losing posture (and potentially consciousness) at a consistent time. During the individual trials there were 11 birds in temperature setting 2, temperature setting 3 was applied to 23 birds and setting 4 was applied to 16 birds. During the group trial, each with 20 birds, 19 groups had temperature setting 2, 19 groups had temperature setting 3 and 12 groups had temperature setting 4. Power calculations based on differences in behaviour durations reported related studies on controlled atmosphere stunning (Abeyesinghe et al. 2007; Lambooij et al 1999 and Gerritzen et al 2004) revealed minimum sample size of 10 birds per treatment group are required in order to achieve an actual power of 0.89. The temperature settings were applied sequentially in accordance with ambient temperature change (setting 4 from 7-12 ^{o}C, setting 3 from 13-18 ^{o}C, and setting 2 from 18-20 ^{o}C) throughout the trial days, resulting in an unbalanced design.

2.4. Trial Procedure

Two different experiments were conducted for individuals and groups. In both, birds were placed in a standard 5-tier US transport module (2.4 x 1.2 x 1.3 m; length x width x height with the second tier from the top being used in the experiment (tier dimensions 1.12m x 1.14m x 0.25m; length x width x height). In the individual trials, the tier was modified by reducing its area by 60% with a soft polystyrene divider (1.12m x 0.36m x 0.25m; length x width x height). This was to minimise damage to the bird when convulsing and to prevent the bird disappearing out of view during LAPS. In the group trials, groups of twenty birds were placed in the allocated tier, without the divider. One hour before the beginning of the trial the birds were transferred to the transport container to mimic lairage. On entering the chamber, birds were filmed for 20s before the LAPS cycle started to determine whether transfer to the LAPS chamber without decompression had an effect on the behaviour of the birds. The focal bird in the group trials was chosen based on proximity to the camera. The trials took place in March, when the temperature in Mississippi varied throughout the day, ambient temperatures

ranged from 9°C-20°C, and temperature settings 2, 3 and 4 were applied. During the trials, the birds were watched in real time on a monitor to check for unexpected behaviour so that the run could be aborted if necessary.

2.5 Behavioural observations

Detailed preliminary observations were carried out to define the ethogram and train the observer before quantitative observations began. Table 1 shows the behaviours that were recorded during LAPS. Description of behaviour categories was adapted from previous work on CAS (Lambooij et al., 1999; Webster and Fletcher, 2001; Gerritzen et al., 2004; Abeyesinghe et al., 2007; Gerritzen, 2007; McKeegan et al., 2007a; McKeegan et al., 2007b; Coenen et al., 2009). Observer XT (Version 12 basic package, live video watching: Noldus Information Technology, Wageningen, the Netherlands) was used to record and analyse the behaviour variables (latencies, bouts and counts) before transferring the data into Excel and R for statistical analysis (R Core team 2014).

2.5. Statistical Analysis

Variables were created relating to the latencies, durations, bout numbers and bout durations (where appropriate) of the behaviours shown in Table 1. Following testing for normality with the Anderson Darling test, using the nortest R package version 1.0-2 (Gross and Ligges, 2012), and checking normality with a histogram of the data, a one-way analysis of variance (ANOVA) or Kruskal Wallis tests were carried out with temperature setting was applied as a factor**.** In individuals, correlations between behavioural parameters and body weight were carried out using Pearson's correlation and Spearman's rank correlation, using the pspearson test R package version 0.3-0 (Savicky, 2014). Where temperature setting did not have an effect, data was pooled for further analysis, but if temperature setting was significant then weight correlations were carried out within each temperature setting. To compare results between individuals and groups, Mann-Whitney U tests and independent

two sample t-tests were used where appropriate. When comparing individuals and groups, if temperature setting had a significant effect, analysis was carried out within temperature setting.

3. Results

3.1. Individual observations

Behaviour in the 20 s before LAPS began was not formally analysed but was generally unremarkable, with the majority of birds sitting. In individuals, 13 birds were seen to exhibit behaviour in addition to sitting; eight birds exhibited some restless behaviour, four showed open bill breathing, four showed mandibulation and one showed headshaking in addition . In groups, nine birds exhibited some restless behaviour, two showed open bill breathing, five showed mandibulation and one showed headshaking. A consistent series of behavioural responses to LAPS were observed: ataxia, loss of posture, clonic and tonic convulsions and leg paddling. The behaviours observed and the proportion of birds carrying out those behaviours are summarised in Tables 2 and 3. Descriptive statistics in the text are mean ± SD.

Ataxia was observed in all birds and the latency to ataxia was 57.3 ± 11.5 s (Table 2). As shown in Figure 1, temperature setting had a significant effect on the latency to ataxia (P = 0.004, One-way ANOVA, $F_{2,47}$ = 6.142). At temperature setting 2, applied when ambient temperatures were warmest, ataxia was earlier than at settings 3 and 4. The mean duration of ataxia was 23.4 ± 16.2s (Table 2). Bird weight was positively associated with the duration of ataxia (n = 50, P = 0.015, Spearman's Correlation, rho = 0.341, Figure 2) and a significant correlation remained following removal of an outlier, suggesting that this correlation is not artefactual. Loss of posture was observed in all birds with a mean latency of 80.7 ± 17.7s (Table 2). Slow wing flapping was observed in 41/50 birds, with a mean latency of 129.6 ± 45.7 s (Table 2). Temperature setting had a significant effect on latency to slow wing flapping (P = 0.003, One-way ANOVA, $F_{2,38}$ = 6.989), which was increased at temperature setting 3 compared with settings 2 and 4 (Figure 1). The mean total time spent slow wing flapping

239 was 8.8 ± 4.4 s. The mean duration of each slow flapping bout was 4.7 ± 2.4 s and the number of
240 bouts ranged from 1-5 (Table 3).
241
242 Clonic convulsions occurred with a mean latency of 110.5 ± 37.6 s (Table 2) and mean duration of
243 11.4 ± 5.7 s. The number of clonic convulsion bouts (2.5 ± 1.4, range 1-7; Table 3) was affected by
244 temperature setting (P = 0.030, Kruskal Wallis, X^2 = 7.015, df = 2), being higher at temperature
245 setting 2 and reducing a stepwise fashion (Figure 3). Tonic convulsions had a mean latency of 117.4
246 ± 28.8 s (Table 2). Time to onset of tonic convulsions was affected by temperature setting (P = 0.026,
247 One-way ANOVA, $F_{2,46}$ = 3.975), where exposure to LAPS at temperature setting 3 induced tonic
248 convulsions faster than at settings 3 and 4 (Figure 1). Tonic convulsions had a mean bout length of
249 5.9 ± 4.3 s and total duration of 19.0 ± 11.7 s (Table 3). The number of bouts of tonic convulsion (3.9
250 ± 2.3) was significantly different between temperature settings (P = 0.037, Kruskal Wallis, X^2 =
251 6.575, df = 2), with birds exposed to temperature setting 4 exhibiting fewer bouts than those at
252 settings 2 and 3 (Figure 3). Leg paddling was observed in 42/50 birds, with a mean latency of 161.2
253 ± 29.6 s (Table 2). Temperature setting affected the total duration of leg paddling (P = 0.028, One-
254 way ANOVA, $F_{2, 39}$ = 3.935, df = 2) with birds at temperature setting 2 spending less time leg
255 paddling than individuals at temperature settings 3 and 4, representing a stepwise trend (Table 2).
256 Leg paddling bout durations were also affected by temperature setting (P = 0.019, Kruskal Wallis, X^2
257 = 7.960, df = 2) in the same way. Becoming motionless was observed in 49/50 birds (because one
258 bird moved out of sight) with a mean latency of 199.4 ± 21.3 s (Table 2).
259
260 Headshaking was observed in 38/50 birds, with mean latency of 50.8 ± 38.3 s and a mean number of
261 3.3 ± 2.8 (range 1-11). Open bill breathing was observed in 37/50 birds with a latency ranging from
262 4.3-187.9 s and 2.4 ± 2.1 bouts per bird. Mandibulation was observed in 16/50 birds with a mean
263 latency of 44.5 ± 31.6 s and 2.1 ± 1.5 bouts per bird (range 1-5). Eighteen birds reacted with alerting
264 behaviour ('notice') at the onset of LAPS. Pecking the environment was observed in 11/50 birds with
265 a mean latency of 55.8 ± 12.5 s and 2.6 ± 2.4 pecks per bird. Jumping was observed in 12 birds with a
266 mean latency of 112.8 ± 40.1 s and 1.7 ± 0.9 jumps per bird (range of 1-3). Four birds jumped before

loss of posture; three birds jumped once and one jumped a total of three times. The mean time to loss of jaw tension was 103.8 ± 34.4 s. The latter behaviours were too rare to analyse in relation to weight and temperature.

3.2. Group observations

The series of behavioural responses observed in groups was the same as individuals, and these are summarised in Tables 4 and 5. Accurate observation of a focal bird in a group of 20 was challenging; on several occasions birds could not be seen temporarily because they moved behind other birds (average total time out of view was 42.8 s (where either the wings, head and/or whole body was out of view). Eleven focal birds went completely out of view (average duration 18.5 ± 15.7 s). Latency to ataxia in groups had a mean of 58.3 ± 8.9 s (Table 4). The duration of ataxia could only be noted in 40 birds because of lack of loss of posture (9 birds) and ataxia data (1 bird) and was 21.9 ± 10.4 s. Loss of posture was reliably established in 41/50 birds with a mean latency of 80.4 ± 11.1 s (range 50.0-117.8 s).

Mean latency to slow wing flap was 104.5 ± 28.5 s (Table 4) with a mean duration of 6.7 ± 4.4 s. The mean total number of bouts of slow wing flapping was 2.4 ± 1.5 (range 1-6) (Table 5). Forty-three birds exhibited clonic convulsions with a mean latency of 128.2 ± 38.3 s (Table 4). Temperature setting affected latency to clonic convulsions (P = 0.036, Kruskal Wallis, X^2 = 6.674, df = 2), which was increased at temperature setting 2, compared with settings 3 and 4 (Figure 4). Tonic convulsions had an mean onset of 129.4 ± 35.7 s (Table 4) and lasted 10.1 ± 6.9 s with a 3.5 ± 2.0 of bouts per bird (range 1-8) (Table 5). The number of bouts of tonic convulsions were higher at temperature setting 2 followed by temperature setting 3 and then temperature setting 4 (P = 0.026, Kruskal Wallis, X^2 = 7.262, df = 2) (Figure 5). Leg paddling had a mean latency of 162.0 ± 27.0s and a mean duration of 9.0 ± 5.4 s (Table 4). Becoming motionless was observed in 48 of the birds (the other two were out of view) with an average onset of 207.5 ± 12.0 s (Table 4), and was affected by temperature setting (P = <0.001, Kruskal Wallis, X^2 = 15.184, df = 2) motionless happened latest at setting 2, followed by setting 3 and 4 (Figure 4).

295

296 Headshaking was observed in 38/50 of focal birds in groups, with mean latency of 58.5 ± 29.6 s and
297 3.5 ± 2.9 times per bird (range 1-11). Open bill breathing was observed in 45/50 group birds with a
298 mean latency of 64.4 ± 29.3 s and more bouts per bird at temperature setting 2 compared to 3 and 4 (*P*
299 = 0.009, Kruskal Wallis, $X^2 = 9.337$, df = 2). Mandibulation was observed in 33/50 birds in groups
300 with a mean onset of 58.0 ± 43.7 s and mean number of bouts of 2.4 ± 2.7. Twenty-three birds
301 showed 'notice' behaviour at the onset of LAPS. Only two individuals pecked the environment and
302 those that pecked did so only once. Fifteen birds jumped during LAPS, with the average jump
303 occurring 132.5 ± 39.1 s after LAPS onset. The mean number of jumping bouts was 2.1 ± 1.2. Loss of
304 jaw tension was only observed in 4 birds with a latency of 95.7 ± 11.8 s, but we note that this
305 response was particularly difficult to observe in groups. The latter behaviours were too rare to analyse
306 in relation to weight and temperature.
307

308 *3.3. Comparisons between individuals and groups*

309 Some differences were noted between individuals and groups. However, these differences must be
310 interpreted with caution because some of the group data was not as reliable as the individual data due
311 to birds being frequently out of view. Latency to ataxia and slow wing flapping was shorter in
312 groups, while latency to headshake and show clonic convulsions were increased compared to
313 individuals (Table 6). Further, total duration and bout duration of slow wing flapping, clonic and
314 tonic convulsions were also shorter in groups than individuals, while the number of clonic convulsion
315 bouts and open bill breathing bouts were higher in groups (Table 6).

316 **4. Discussion**

317 This study provides the first comprehensive behavioural data for broilers undergoing LAPS, and a
318 consistent series of responses were observed. The data provide a basis for comparison with related
319 hypoxic killing methods such as CAS, and in general the same range of behaviour patterns was
320 apparent - ataxia, loss of posture, clonic and tonic convulsions, leg paddling and becoming

motionless. As has been noted in previous studies on CAS (e.g. Abeyesinghe et al., 2007), there were qualitative and (to a greater extent) quantitative variations in behavioural responses to LAPS. These differences were not accounted for by bodyweight (where analysis was possible) and presumably relate to other factors which remain to be identified but could include physiological traits such as lung capacity and air sac volume and response of the brain to anoxia. The individual variation seen in broilers is analogous to the results of studies in man of response to hypobaric chambers during pilot training which revealed a high degree of individual variation between the range of symptoms' and signs experienced (Woodruff and Webb, 2011). The weight of the birds used in this study ranged from 2.5 Kg to more than 4Kg, thus while the mean reflected larger US boiler weights, there was some overlap with broilers weights usually seen in the Europe. Bird weight correlated with only one behavioural variable (duration of ataxia), so it appears that bird weight has a minimal effect and this concurs with commercial experience that bird size does not have a significant impact on the process.

Headshaking, mandibulation and open bill breathing (or other forms of respiratory disruption) have been observed in many studies of poultry undergoing CAS with both hypercapnic and inert anoxic gas mixtures and these were also seen with LAPS (in 76%, 32% and 74% of birds respectively). Experiments involving exposure to anoxia with inert gases provide the most relevant comparisons to LAPS (though note that in most cases CAS studies involve immersion in the gas and not gradual replacement of air), and various authors have reported headshaking in response to Argon and Nitrogen (Lambooij et al. 1999; Gerritzen et al., 2000; Webster and Fletcher 2001; McKeegan et al., 2007a; Abeyesinghe et al., 2007). The mean number of headshakes observed in response to LAPS was 3, and this is intermediate between previous reports of 0.5 and one for Argon and Nitrogen respectively (McKeegan et al., 2007a) and nine for Argon (Gerritzen et al. 2000). Headshaking has been interpreted as an aversive reaction to carbon dioxide (Raj, 1996) but this does not explain its occurrence in response to inert gases. Headshaking may indicate disorientation, discomfort, respiratory distress (Webster and Fletcher 2001) or arousal (Hughes 1983) but it was not seen in all birds which we might expect if certain sensations causing headshaking were a direct consequence of undergoing LAPS. There are concerns that expansion of gases in body tissues or sinuses may cause

discomfort or pain during LAPS. Future work with analgesic intervention could help to determine if the headshaking seen in the early part of LAPS induction is pain related. Mandibulation was observed in a minority of birds during LAPS; this behaviour has also been observed previously in response to Argon and Nitrogen (Webster and Fletcher 2001; McKeegan et al 2007a) which suggests that the reduction in oxygen or another environmental factor is stimulating gustatory or trigeminal receptors in the mucosal membrane of the birds. The relevance of this behaviour to welfare is unclear; during LAPS it may also serve the function of equalising pressure between the ears and oral cavity via the Eustachian tubes.

Open bill breathing was recorded in three quarters of the birds and this may indicate some dyspnoea (respiratory discomfort), similar to CAS. Open bill breathing has been interpreted as an indication of breathlessness in birds (Gerritzen et al., 2004), and breathlessness in mammals was recently defined as a negative affective experience relating to respiration with multiple qualities (Beausoleil and Mellor, 2015). In humans, these include respiratory effort, air hunger (increased urge to breath) and chest tightness (Beausoleil and Mellor, 2015), though it is not clear whether these all apply to birds, which have a unique respiratory system of unidirectional air flow through the lungs and multiple air sacs. It has been suggested that anoxia results in air hunger in humans (Moosavi et al., 2003), and Beausoleil and Mellor (2015) suggest that this may have the greatest potential to compromise welfare compared to other forms of respiratory discomfort. There is evidence that some dyspnoea occurs in all CAS stunning mixtures that have been investigated, including Argon and Nitrogen (e.g. Gerritzen et al., 2004; Abeyesinghe et al., 2007). During LAPS, birds exhibited an average of 2.4 bouts of open bill breathing, which is very close to a previously reported value for exposure to Argon (2.25 bouts, McKeegan et al., 2007a) but less than for a hypercapnic mixture in the same study (13 bouts). Given that headshaking, mandibulation and open bill breathing are all seen during exposure to anoxic gases (normobaric hypoxia) as well as during LAPS (hypobaric hypoxia); it is difficult to conclude whether they are a response to hypoxia or decompression, or both. It is also difficult to determine if such signs are part of the birds' normal physiological response to hypoxia or evidence of pain, for which there is

no direct indicator (EFSA, 2013). Indeed, a few birds exhibited mandibulation, head shaking and open bill breathing before LAPS began.

Loss of posture has been widely interpreted as a proxy for loss of consciousness (Gerritzen et al., 2004, EFSA 2013) and during LAPS loss of posture occurred on average at 80.7s in individually killed birds and at 80.4s in group killed birds. In previous studies on CAS, immersion in inert anoxic gases has tended to result in a much more rapid loss of posture (e.g. 15.6s in Argon, Lambooij et al., 1999). The gradual nature of LAPS means that birds experience a longer induction and therefore there is a greater time period where they could potentially experience negative welfare. However, obvious escape behaviours that have been seen during CAS (e.g. McKeegan et al., 2007a) were not seen during LAPS and previous work in which ECG data was collected during LAPS (McKeegan et al., 2013), showed no evidence of heart rate increase during induction (albeit from an elevated baseline). McKeegan et al (2013) suggested a time to loss of consciousness of 40s, based on spectral analysis of EEG recordings during LAPS. This does not match the observations in the current study. There are a number of possible explanations for this discrepancy; the most likely is that because the birds undergo LAPS in complete darkness, the EEG response was confounded with sleep-like waves that are induced by simulated eye-closure. Another factor to consider is that the EEG study took place under different ambient conditions (high summer temperatures of 40 ^{o}C) and therefore a different temperature setting and decompression curve was applied. Experiments recording EEG output and behaviour within the same bird during LAPS are necessary to generate a corroborated time to loss of consciousness on an individual bird basis.

Clonic convulsions and tonic convulsions are commonly seen in gas stunning (reviewed in Raj, 2006) and were also seen in LAPS. During LAPS, convulsions always occurred after loss of posture, indicating that birds are in an unconscious state (Gerritzen et al., 2004, EFSA 2013). A previous behavioural study on LAPS determined that clonic wing flapping was a major cause of wing damage in birds killed by LAPS (Vizzier-Thaxton et al., 2010) but this is not a welfare issue as the self-inflicted injury occurs when the affected bird is unconscious. However, when birds are killed in

404 groups, as is done commercially, it may be possible for birds that are still in a conscious state to be
405 disturbed or even injured by other birds wing flapping. In the current study, the total duration of
406 clonic convulsions and slow wing flapping in groups was 14s which is similar to the 15.1s determined
407 by Vizzier-Thaxton et al (2010) who also made observations in groups. The wing flapping duration
408 was slightly higher for individually killed birds in the current study, with a 20.2s total duration. These
409 figures are very similar to those previously reported for anoxic CAS (17.5s in Argon and 15.7s in
410 Nitrogen, McKeegan et al 2007a).
411
412 Although placing birds individually in the LAPS chamber maximised visibility of their behavioural
413 responses, they are likely to have experienced some isolation stress (Cheng et al, 2003) which may
414 have affected the results. While most birds sat in a resting position before LAPS, some did show
415 restless behaviour (though this was observed in both groups and individuals). Applying LAPS to
416 groups of 20 birds was commercially relevant, but obscuration of the focal bird by neighbours made
417 observations difficult and the resulting data less accurate. Several significant differences between
418 individuals and focal birds in groups were found, but in almost every case these were in the same
419 direction – individuals had shorter latencies, longer durations and more bouts than groups. Increased
420 latencies are probably because in groups, some birds may have been out view the first time the
421 behaviour happened, and decreased durations/bouts are also likely to be due to behaviour sometimes
422 not being visible. Therefore, these differences are probably not meaningful and instead reflect the
423 limitations of accuracy of the group observations. Abeyesinghe et al (2007) reported differences in
424 responses to gas stunning mixtures between individuals and groups and concluded that these were at
425 least partially due to difficulties with observations in groups. In the current study, there were two
426 exceptions to the normal group effect; latency to show slow wing flapping was reduced in groups, and
427 this may be a genuine group effect, with wing flapping being caused by disturbance by neighbouring
428 birds. Focal birds in groups also showed more open bill breathing, but the reason for this is not clear
429 since the number of birds in the chamber should not significantly affect oxygen availability and other
430 measures relating to hypoxia such as time to loss of posture were not different between groups and

individuals. In the future, observing birds undergoing LAPS in small groups such as pairs or triplets may be a good way to improve visibility while eliminating isolation stress.

The automatic temperature settings resulted in slightly different pressure curves being applied, but the decompression rate in the first 67s of LAPS was never altered. In this study, only three of the six available settings were applied (due to the limited ambient temperature range). While there were some effects of temperature setting on behaviour, these were not consistent and many did not show stepwise trends making their interpretation difficult. Time to ataxia did show a stepwise trend, being greatest in colder conditions which may relate both to greater air density and lower humidity in cold air (with consequently increased oxygen availability) and physiological responses to air temperature affecting oxygen exchange. Conversely, time to motionless in groups was faster at colder temperatures, which may indicate overcompensation in the decompression curve in the later part of the LAPS cycle.

To be humane, stunning methods should produce insensibility with minimum welfare concerns (Joseph et al., 2013). Like CAS, LAPS has many advantages for commercial poultry slaughter, including avoiding live shackling and ensuring every bird is stunned. The behavioural data presented here suggest that LAPS is largely equivalent to anoxic gas stunning in the range of behaviours it elicits, except that due to the gradual nature of the decompression, birds take longer to lose consciousness. In the conscious phase, birds exhibit behaviour which has been previously associated with controlled atmosphere stunning, namely mandibulation, headshaking and open bill breathing, but not more so than in CAS. This behavioural evidence suggests that LAPS is a humane method for stunning poultry. Further work is required to understand the stimuli that give rise to behaviours that may reflect reduced welfare and to corroborate behavioural indicators of time to loss of consciousness with EEG measurements on an individual bird basis.

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Table 1. Behavioural categories recorded during LAPS

Behaviour	**Description**
Notice	Alert/restless movements of the head and/or restless movements of the body.
Mandibulation	Repetitive and rapid opening and closing of the bill.
Headshake	Rapid lateral head movement.
Open bill breathing	Breathing with bill open, with or without neck extension.
Ataxia	Apparent dizziness, staggering, swaying of body and/or head, attempts to stand/sit or flaps wings to try and regain balance.
Loss of posture	Unable to regain/maintain a controlled posture.
Clonic convulsion	Rapid/vigorous movement of the wings, a new bout was defined as following a pause of at least one second.
Tonic convulsion	Uncontrolled twitching (visible muscular spasms within the body). A new bout was defined as following a pause of at least one second.
Slow wing flapping	One short burst or prolonged slow/moderate movement of the wings, occurring without any twitching of the body. A new bout was defined by a pause of one second.
Leg paddling	Involuntary, usually alternating, leg movements in the air or towards the ground depending on the body position of the bird. Leg paddling can also be determined by an alternating upwards and downwards movement of the body if bird is lying sternal. A new bout was defined by a pause of one second. A new bout was defined by a pause of one second.
Loss of jaw tension	Bill open for more than 2s without deep breathing and/or neck extension.
Jump	Explosive movement from a sitting/lying position to stand and then immediately back to sitting/lying position.
Peck	Moving head backwards and forwards in a pecking motion.
Motionless	No discernible body or breathing movements.
Sitting	Legs underneath the body cavity and wings relaxed against body wall.
Standing	Standing with the body fully or partly lifted off of the ground.
Lying	Lying once posture is lost and not perceived to be purposefully controlling posture.
None/ not seen/ unsighted	No noticeable body movements, wing movements, leg movements or the bird was completely out of view.

Table 2. Summary of behavioural results from the individual trials, showing the percentage of birds exhibiting each behaviour, and mean latency (Lat) and range, mean total duration (TD) and range, and results of one way ANOVA/Kruskal Wallis analysis for the effect of temperature setting.

Behaviour	**Birds (%)**	**Mean (±SD) Lat (s)**	**Range Lat (s)**	***P* value Lat**	**Mean (±SD) TD (s)**	**Range TD (s)**	***P* value TD**
Ataxia	100	57.3 (11.5)	17.8-77.2	0.004	23.4 (16.2)	5.8-105.2	0.285
Loss of posture	100	80.7(17.7)	58.8-182.5	0.072	-	-	-
Clonic	98	110.5 (37.6)	63.3-208.2	0.955	11.4 (5.7)	1.3-25.5	0.965
Tonic	98	117.4 (28.8)	73.9-185.3	0.026	19.0 (11.7)	1.3-61.1	0.303
Slow wing flap	82	129.6 (45.7)	10.2-209.5	0.003	8.8 (4.4)	0.8-17.7	0.675
Leg paddling	84	161.2 (29.6)	110.3-220.7	0.202	10.1 (6.2)	0.7-26.7	0.028
Motionless	98	199.4 (21.3)	158.2-245.6	0.136	-	-	-
Headshaking	76	50.8 (38.3)	3.3-167.3	0.108	-	-	-
Open bill breathing	74	57.4 (35.8)	4.3-187.9	0.727	-	-	-
Mandibulation	32	44.5 (31.6)	4.4-137.5	0.863	-	-	-

All degrees of freedom (df) = 2. N=50.

Table 3. Summary of behavioural results from the individual trials, showing the percentage of birds exhibiting each behaviour, and mean single bout duration (SBD) and range, mean number of bouts and range, and results of one way ANOVA/Kruskal Wallis analysis for the effect of temperature setting.

Behaviour	**Birds (%)**	**Mean (±SD) SBD (s)**	**Range SBD (s)**	***P* value SBD**	**Mean (±SD) Number of Bouts**	**Range Number of Bouts**	***P* value Number of Bouts**
Clonic	98	5.5 (3.6)	1.3-19.3	0.707	2.5 (1.4)	1-7	0.030
Tonic	98	5.9 (4.3)	1.3-20.4	0.465	3.9 (2.3)	1-12	0.037
Slow wing flap	82	4.7 (2.4)	0.8-11.8	0.674	2.1 (1.1)	1-5	0.190
Leg paddling	84	7.5 (4.6)	0.7-19.7	0.019	1.5 (0.8)	1-3	0.795
Headshaking	76	-	-	-	3.3 (2.8)	1-11	0.827
Open bill breathing	74	-	-	-	2.4 (2.1)	1-10	0.750
Mandibulation	32	-	-	-	2.1 (1.5)	1-5	0.755

All degrees of freedom (df) = 2. N=50

Table 4. Summary of behavioural results from the group trials, showing the percentage of birds exhibiting each behaviour, and mean latency (Lat) and range, mean total duration (TD) and range, and results of one way ANOVA/Kruskal Wallis analysis for the effect of temperature setting.

Behaviour	**Birds (%)**	**Mean (±SD) Lat (s)**	**Range Lat (s)**	***P* value Lat**	**Mean (±SD) TD (s)**	**Range TD (s)**	***P* value TD**
Ataxia	98 / 80	58.3 (8.9)	39.6-78.6	0.520	21.9 (10.4)	4.5-45.0	0.781
Loss of posture	82	80.4 (11.1)	50.0-117.8	0.507	-	-	-
Clonic	86	128.2 (38.3)	66.7-204.6	0.036	7.3 (4.6)	1.0-21.2	0.191
Tonic	84	129.4 (35.7)	77.3-197.2	0.152	10.1 (6.9)	0.9-26.1	0.080
Slow wing flap	92	104.5 (28.5)	63.1-169.9	0.610	6.7 (4.4)	0.4-19.7	0.896
Leg paddling	62	162.0 (27.0)	104.5-207.4	0.228	9.0 (5.4)	1.1-19.1	0.921
Motionless	96	207.5 (12.0)	180.1-235.3	<0.001	-	-	-
Headshaking	76	58.5 (29.6)	4.1-147.8	0.461	-	-	-
Open bill breathing	90	64.4 (29.3)	5.4-162.3	0.380	-	-	-
Mandibulation	66	58.0 (43.7)	2.8-174.2	0.615	-	-	-

All degrees of freedom (df) = 2. N=50.

Table 5. Summary of behavioural results from the group trials, showing the percentage of birds exhibiting each behaviour, and mean single bout duration (SBD) and range, mean number of bouts and range, and results of one way ANOVA/Kruskal Wallis analysis for the effect of temperature setting.

Behaviour	**Birds (%)**	**Mean (±SD) SBD (s)**	**Range SBD (s)**	***P* value SBD**	**Mean (±SD) Number of Bouts**	**Range Number of Bouts**	***P* value Number of Bouts**
Clonic	86	3.0 (1.6)	1.0-7.5	0.464	2.6 (1.6)	1-7	0.213
Tonic	84	2.8 (1.3)	0.9-6.6	0.839	3.5 (2.0)	1-8	0.026
Slow wing flap	92	3.1 (2.4)	0.4-12.6	0.129	2.4 (1.5)	1-6	0.305
Leg paddling	62	6.1 (3.9)	1.1 -17.2	0.220	1.6 (0.8)	1-4	0.212
Headshaking	76	-	-	-	3.5 (2.9)	1-11	0.971
Open bill breathing	90	-	-	-	3.2 (2.6)	1-15	0.009
Mandibulation	66	-	-	-	2.4 (2.7)	1-14	0.559

All degrees of freedom (df) = 2. N=50.

Table 6. Outcome of statistical comparisons between individual and group trials.

Behavioural response		**Outcome of t-test/Mann Whitney U**
Latency	Ataxia	$P = 0.003$, t = -3.418
	Slow wing flap	$P < 0.001$, W = 260
	Headshake	$P = 0.031$, W = 514
	Clonic convulsions	$P = 0.038$, t = 2.257
Total duration	Slow wing flap	$P = 0.025$, W = 1206
	Clonic convulsions	$P < 0.001$, t = 3.856
	Tonic convulsions	$P < 0.001$, W = 1549
Bout duration	Slow wing flap	$P < 0.001$, W = 1388
	Clonic convulsions	$P < 0.001$, W = 1601.5
	Tonic convulsions	$P < 0.001$, W = 1576
Number of bouts	Clonic convulsions	$P = 0.012$, W = 145
	Open bill breathing	$P = 0.028$, W = 36

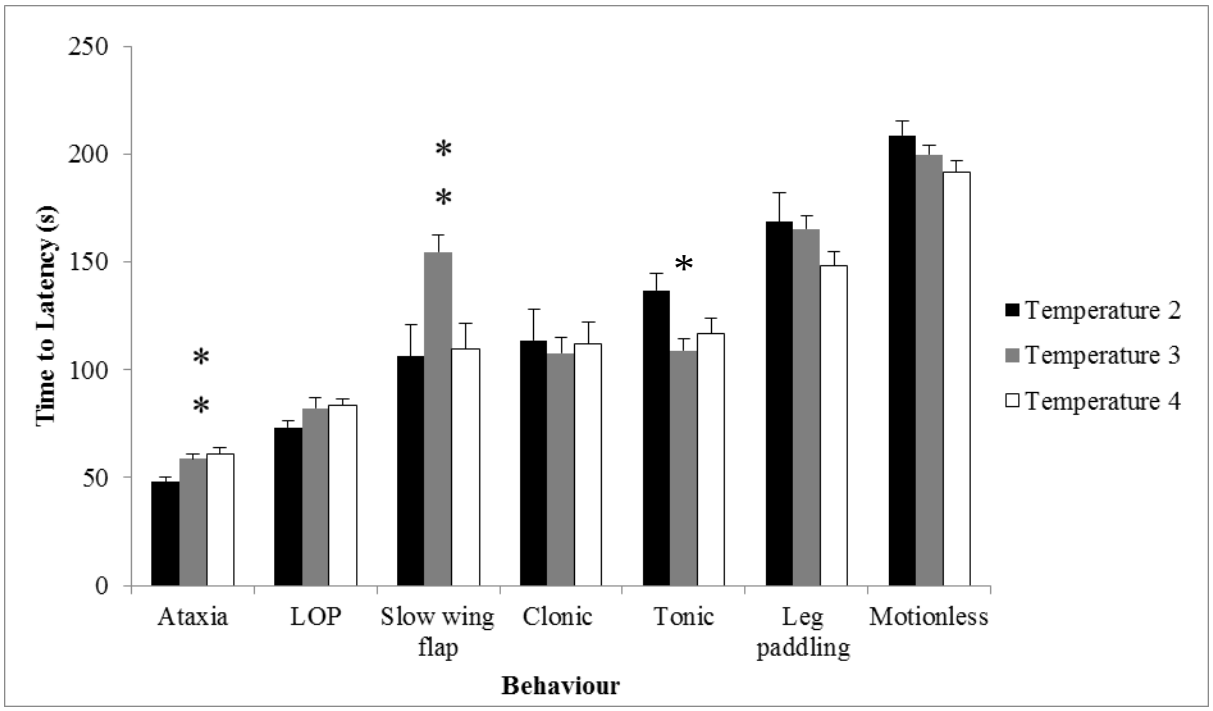

Figure 1. Mean (SEM) time to latency of each behaviour at each temperature setting in individual killed birds. LOP = Loss of posture.
Temperature 2: 11 birds, *Temperature 3*: 23 birds, *Temperature 4*: 16 birds
*=<0.05, **=<0.01, ***=<0.001

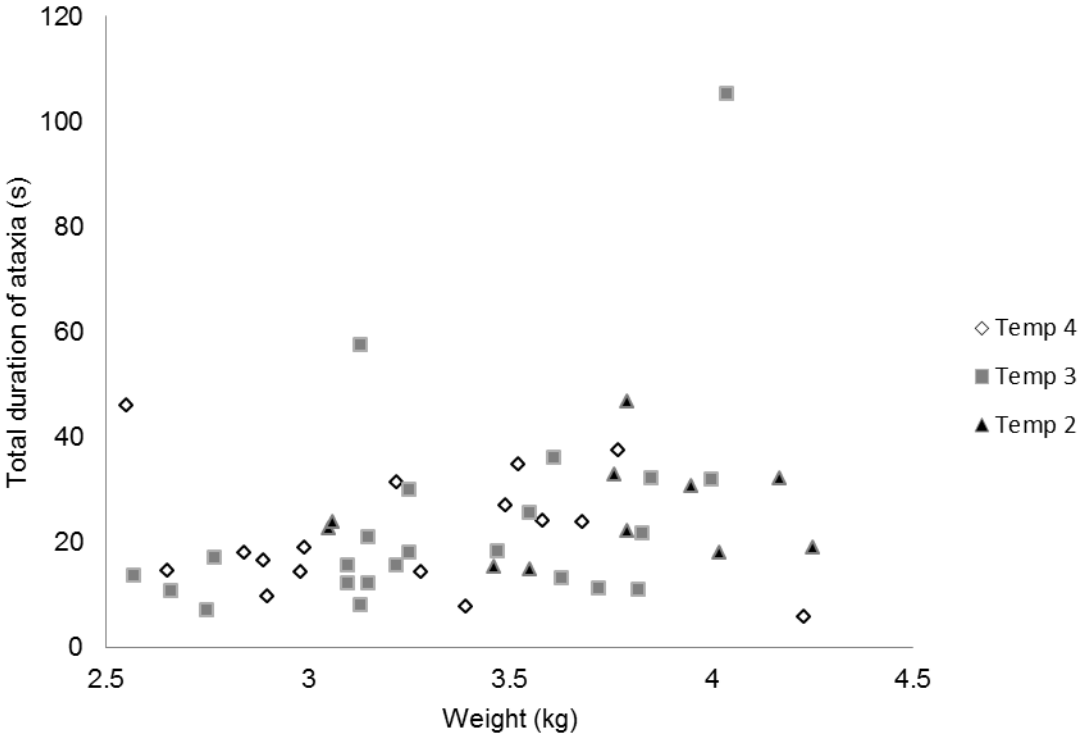

Figure 2. Scatter plot showing the relationship between duration of ataxia and individual bird body weight at each temperature setting; N=50.

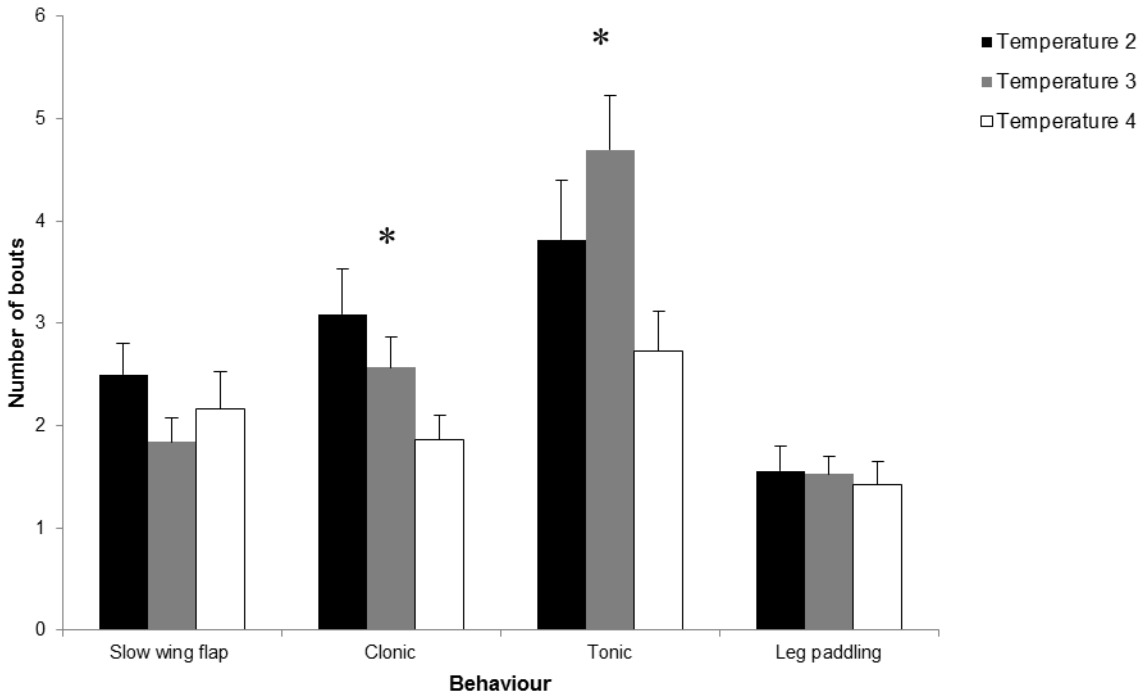

Figure 3. Mean (SEM) number of bouts of each behaviour at each temperature setting in individually killed birds.
Temperature setting 2: 11 birds, *Temperature setting 3*: 23 birds, *Temperature setting 4*: 16 birds
*=<0.05, **=<0.01, ***=<0.001

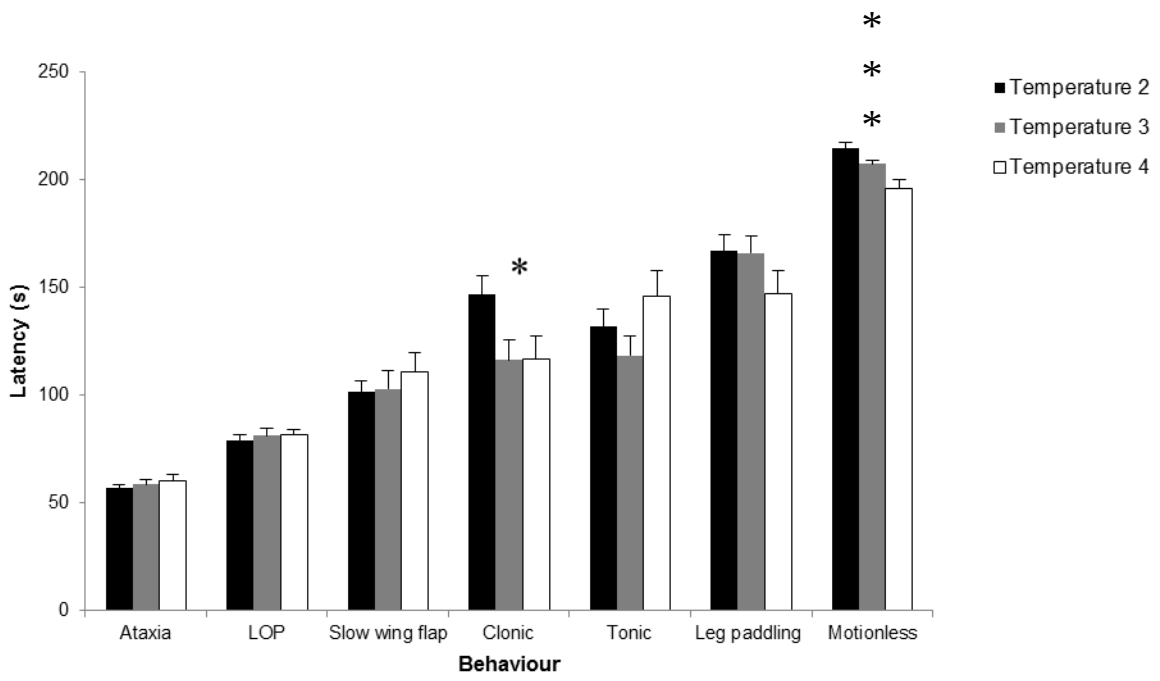

Figure 4. Mean (SEM) latency of each behaviour at each temperature setting in group killed birds. LOP = Loss of posture.
Temperature setting 2: 19 birds, *Temperature setting 3*: 19 birds, *Temperature setting 4*: 12 birds
*=<0.05, **=<0.01, ***=<0.001

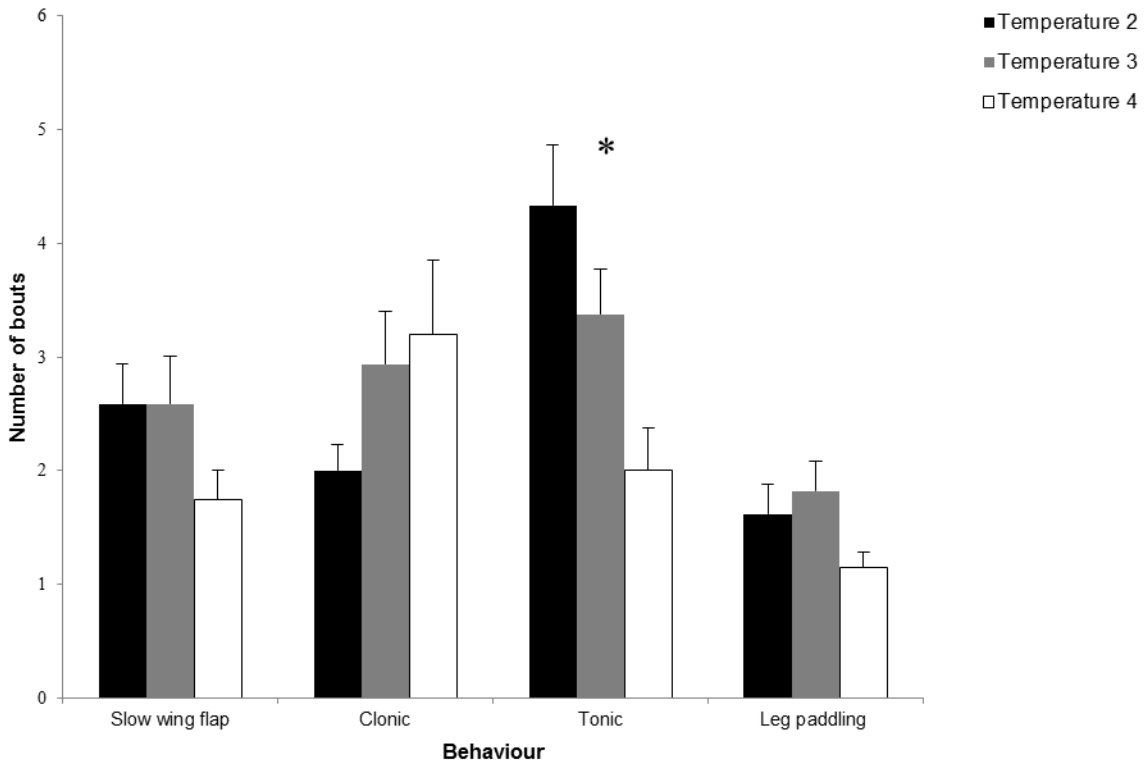

Figure 5. Mean (SEM) number of bouts of each behaviour at each temperature setting in group killed birds.
Temperature setting 2: 19 birds, *Temperature setting 3*: 19 birds, *Temperature setting 4*: 12 birds
*=<0.05, **=<0.01, ***=<0.001